Increased Calcium Levels and Prolonged Shelf Life in Tomatoes Expressing Arabidopsis H^+/Ca^{2+} Transporters[1]

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Here we demonstrate that fruit from tomato (*Lycopersicon esculentum*) plants expressing Arabidopsis (*Arabidopsis thaliana*) H^+/cation exchangers (CAX) have more calcium (Ca^{2+}) and prolonged shelf life when compared to controls. Previously, using the prototypical CAX1, it has been demonstrated that, in yeast (*Saccharomyces cerevisiae*) cells, CAX transporters are activated when the N-terminal autoinhibitory region is deleted, to give an N-terminally truncated CAX (sCAX), or altered through specific manipulations. To continue to understand the diversity of CAX function, we used yeast assays to characterize the putative transport properties of CAX4 and N-terminal variants of CAX4. CAX4 variants can suppress the Ca^{2+} hypersensitive yeast phenotypes and also appear to be more specific Ca^{2+} transporters than sCAX1. We then compared the phenotypes of *sCAX1*- and *CAX4*-expressing tomato lines. The *sCAX1*-expressing tomato lines demonstrate increased vacuolar H^+/Ca^{2+} transport, when measured in root tissue, elevated fruit Ca^{2+} level, and prolonged shelf life but have severe alterations in plant development and morphology, including increased incidence of blossom-end rot. The *CAX4*-expressing plants demonstrate more modest increases in Ca^{2+} levels and shelf life but no deleterious effects on plant growth. These findings suggest that CAX expression may fortify plants with Ca^{2+} and may serve as an alternative to the application of $CaCl_2$ used to extend the shelf life of numerous agriculturally important commodities. However, judicious regulation of CAX transport is required to assure optimal plant growth.

Calcium (Ca^{2+}) plays a fundamental role in plant membrane stability, cell wall stabilization, and cell integrity (Hirschi, 2004). Reduced Ca^{2+} in edible plant tissues negatively impacts total yield. Plant tissues low in Ca^{2+} are more susceptible than tissues with normal Ca^{2+} levels to some parasitic diseases during storage (Marschner, 1995). This is of particular concern in the case of fleshy fruits with their typically low Ca^{2+} levels. Application of Ca^{2+} to soils seems to be of questionable value (Lester and Grusak, 1999; Lopez-Lefebre et al., 2001); however, supplemental Ca^{2+} applied immediately before or just after harvest has been shown to maintain cell turgor, plasma membrane integrity, and fruit firmness and extend storage life (Gerasopoulos et al., 1996; Miklus and Beelman, 1996). In these treatments, application of Ca^{2+} increases total Ca^{2+} levels in the fruits by 20% to 40%; however, these procedures are labor intensive and often result in tissue damage and fungal infections.

The problems associated with low plant Ca^{2+} levels can be attributed to soil problems. For example, Ca^{2+} deficiencies are favored by very low soil pH and on soils high in magnesium and potassium. Probably the most recognizable Ca^{2+} deficiency is blossom-end rot (BER) of tomato (*Lycopersicon esculentum*) fruits, which is induced by water stress (Bennett, 1993; Ho and White, 2005). At the time of fruit set, cells at the blossom end of fruits are injured. This is caused by insufficient Ca^{2+} translocation resulting in a dry-rot area on the expanding fruit.

One molecular-genetic approach to alter the Ca^{2+} levels in plants is to engineer high expression of Ca^{2+} transporters and Ca^{2+}-binding proteins (Wyatt et al., 2002; Hirschi, 2004). Increased expression of a modified vacuolar Ca^{2+} antiporter, cation exchanger 1 (CAX1), in plants causes dramatic increases in Ca^{2+} when compared to vector control plants (Hirschi, 1999; Park et al., 2004, 2005). In a similar fashion, expression in tobacco (*Nicotiana tabacum*) of a wheat (*Triticum aestivum*) cation transporter LCT1 increases shoot Ca^{2+} levels (Antosiewicz and Hennig, 2004). Recent findings suggest that the corresponding changes in plant growth and development depend on the levels of transporter activity and the particular plant being modified.

The CAX1 cDNA that has been expressed in various plants was originally cloned through a yeast

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(*Saccharomyces cerevisiae*) suppression screen (Hirschi et al., 1996). We have identified a full-length CAX1 cDNA (previously termed long-CAX1) that is identical to the original clone except that it encodes a protein with an additional 36 amino acids at the N terminus (Pittman and Hirschi, 2001). This CAX1 cannot transport Ca^{2+} in a yeast biochemical assay, and we have shown that the N terminus functions as a regulatory region (Pittman et al., 2002). The original CAX1 is a partial-length cDNA that is deregulated for H^+/Ca^{2+} antiport and is now termed N-terminally truncated CAX 1 (sCAX1). It is this activated form of CAX1 that when expressed in tobacco, carrots (*Daucus carota*), and potatoes (*Solanum tuberosum*) results in increased Ca^{2+} accumulation (Hirschi, 1999; Park et al., 2004, 2005). Recent work in yeast suggests that full-length CAX transporters may have some weak H^+/Ca^{2+} transport capabilities (Cheng et al., 2005). In addition, various domains within the CAX transporters have been identified that confer substrate specificity. For example, the Ca^{2+} domain of CAX1 appears to be necessary for Ca^{2+} transport (Shigaki et al., 2001). An unexamined means of marginally increasing activity of H^+/Ca^{2+} antiporters may be to express the entire CAX open reading frame or mutant CAX variants in plants.

Arabidopsis (*Arabidopsis thaliana*) appears to have up to 12 putative CAX transporters (Mäser et al., 2001). Like sCAX1, sCAX2 and sCAX4 can function in yeast as H^+/Ca^{2+} exchangers (Hirschi et al., 1996; Cheng et al., 2002; Pittman et al., 2004b). Our preliminary biochemical analysis of sCAX1 and sCAX2 suggests transport of various metals (Mäser et al., 2001; Shigaki et al., 2003; Pittman et al., 2004b). However, the specific transport properties of numerous CAX transporters, including CAX4, have not been previously examined. Hence, efforts to specifically alter the Ca^{2+} levels in plants may be more efficient utilizing regulated expression of CAX transporters other than sCAX1.

Our primary focus here was to evaluate the potential for increasing the Ca^{2+} levels of tomatoes through expression of Arabidopsis H^+/Ca^{2+} transporters and its potential impact on plant growth and development. Herein, utilizing yeast assays, we have characterized the transport properties of variants of CAX4. We also compare and contrast the effect of sCAX1 and CAX4 expression in tomato as a method to determine if utilizing full-length CAX expression may be a means to reduce deleterious phenotypes. This study suggests that modulation of Ca^{2+} transporters could make an important contribution toward increasing the value of various agriculturally important crops.

RESULTS

Transport Activity and Cation Selectivity Comparisons of CAX4 Variants

When expressed in yeast, the N-terminal region of CAX transporters acts as an autoinhibitory domain for H^+/Ca^{2+} transport activity (Pittman et al., 2002, 2004b). To further examine the properties of CAX4 transporters with (CAX) and without (sCAX) the putative N-terminal autoinhibitory domain, we expressed these plasmids in a yeast strain deficient in vacuolar Ca^{2+} transporters and lacking functional calcineurin (Cunningham and Fink, 1996; Hirschi et al., 1996). This strain (K667) lacks the endogenous vacuolar Ca^{2+}-ATPase PMC1 and vacuolar H^+/Ca^{2+} antiporter VCX1 and thus is defective in vacuolar Ca^{2+} transport, making it unable to grow on high Ca^{2+} media (Cunningham and Fink, 1996). We examined the growth of yeast cells expressing empty vector control, *sCAX1*, and full-length *CAX1* and *CAX4* in media containing 50 mM $CaCl_2$. Under these semipermissive conditions, the vector control and *CAX4*-expressing strains grew poorly; however, the full-length *CAX1* and truncated *sCAX1*-expressing cells suppressed the Ca^{2+} sensitivity, although the suppression by CAX1 was weaker than sCAX1 (data not shown). We then tested those constructs and CAX4 variants (Cheng et al., 2002) on higher Ca^{2+}-containing media (100 mM $CaCl_2$). One chimeric CAX4 construct has a 37-amino acid N-terminal truncation and contains the 9-amino acid CAX1 Ca^{2+} domain (Shigaki et al., 2001; sCAX4-9), while the other form contains a triple copy of the epitope tag hemagglutinin (HA) fused to the N terminus of full-length *CAX4* (HA-CAX4). As shown in Figure 1A, CAX4 variant-expressing cells can strongly suppress the Ca^{2+} sensitivity in a similar manner to sCAX1.

The Ca^{2+} transport activities of CAX4 and sCAX4 are too weak and cannot give measurable $^{45}Ca^{2+}$ transport in yeast (Cheng et al., 2002; data not shown), so to further infer the transport properties of CAX4, competition transport experiments were performed using the deregulated CAX4 variants HA-CAX4 and sCAX4-9. This allowed us to compare cation selectivity between sCAX1 and these CAX4 variants. ΔpH-dependent 10 μM $^{45}Ca^{2+}$ uptake was measured at a single 10-min time point into yeast vacuolar-enriched membrane vesicles isolated from K667 strains expressing *sCAX1*, *sCAX4-9*, and *HA-CAX4*. Ca^{2+} uptake (10 μM) determined in the absence of excess nonradioactive metal (control) was compared with uptake determined in the presence of two concentrations (10× and 100×) of nonradioactive metals $CaCl_2$, $MnCl_2$, $CdCl_2$, $ZnCl_2$, $NiCl_2$, NaCl, and KCl (Fig. 1B). Inhibition of Ca^{2+} uptake by nonradioacitve Ca^{2+} was used as an internal control, and as expected Ca^{2+} uptake by each CAX transporter was strongly inhibited by excess Ca^{2+}. Nonradioactive Ca^{2+}, particularly the 10× concentration, did not completely inhibit Ca^{2+} uptake, further highlighting the low Ca^{2+} affinity of the CAX transporters. Ca^{2+} uptake by sCAX1 and sCAX4-9 was inhibited by Cd^{2+}, Na^+, and K^+, but this inhibition was only significant at the higher concentrations. Interestingly, HA-CAX4 Ca^{2+} transport was not inhibited by any of the metals tested (Fig. 1B). These results indicate that CAX4 may be more specific for Ca^{2+} than

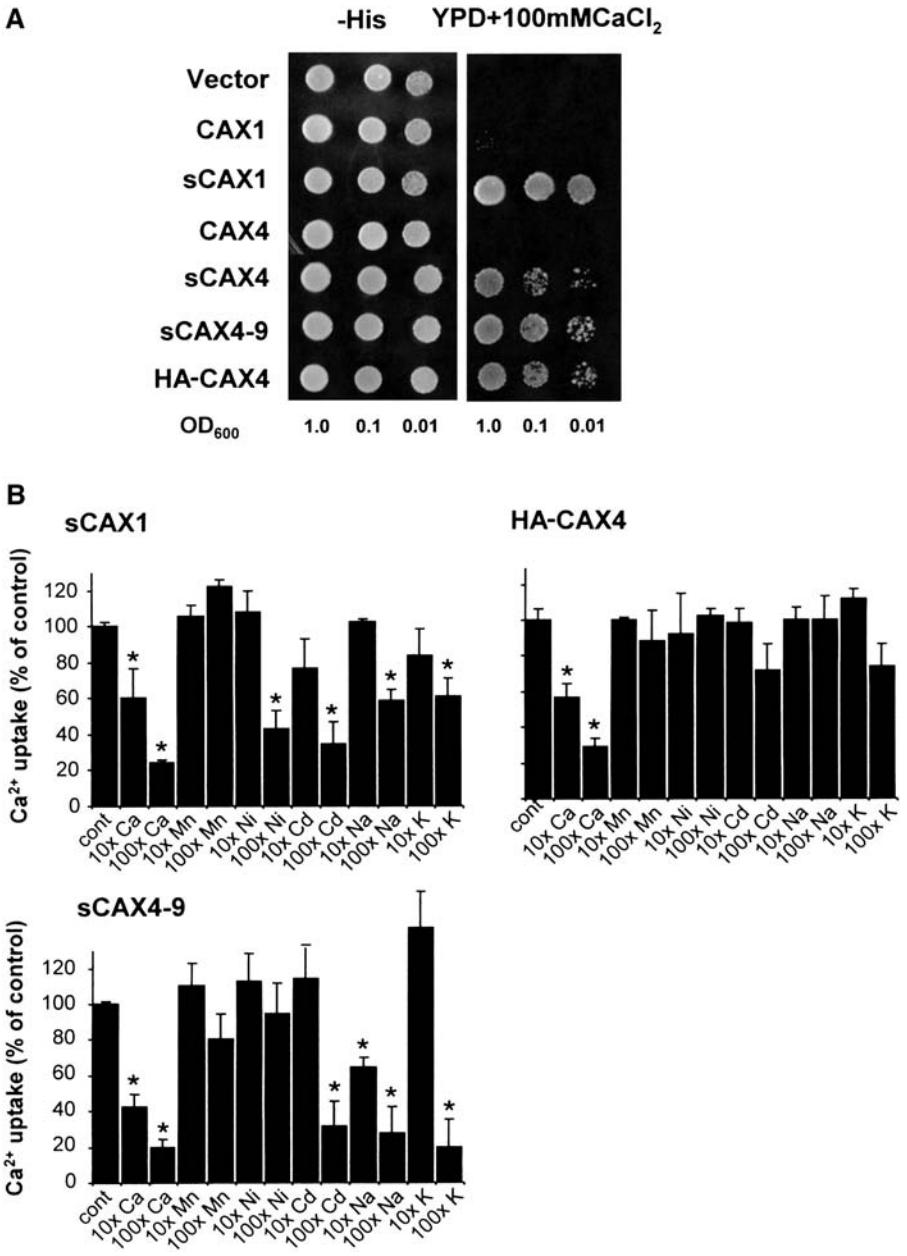

Figure 1. Substrate characteristics of CAX4 variants expressed in yeast. A, Suppression of Ca^{2+} sensitivity of the *pmc1 cnb vcx1* yeast mutant (K667) by CAX1, sCAX1, CAX4, sCAX4, sCAX4-9, and HA-CAX4. Saturated liquid cultures of K667 containing various plasmids were diluted to the cell densities, as indicated, and then spotted onto selection medium (−His) and yeast-extract peptone dextrose (YPD) medium containing 100 mM $CaCl_2$. B, Inhibition of Ca^{2+} uptake by sCAX1, HA-CAX4, and sCAX4-9 into yeast vacuolar membrane vesicles in the presence of other metals. ΔpH-dependent uptake of 10 μM $^{45}Ca^{2+}$, estimated as the difference between uptake with and without 5 μM FCCP, was measured in the absence (control) or presence of 10 × (100 μM) or 100 × (1 mM) of nonradioactive $CaCl_2$, $MnCl_2$, $CdCl_2$, $ZnCl_2$, $NiCl_2$, NaCl, or KCl after 10 min. Ca^{2+} uptake values are shown following subtraction of the FCCP background values and expressed as percentages of the control in the absence of any excess nonradiolabeled metals. The data represent means of two to four replications from two to three independent membrane preparations, and the bars indicate SE. An asterisk indicates significant difference from the control ($P \geq 0.001$).

sCAX1 or the sCAX4 variants that contain the Ca^{2+} domain of CAX1.

CAX Expression in Tomato

The phenotypes of transgenic plants expressing *sCAX1*, in conjunction with the biochemical properties of sCAX1 in yeast, suggest that expression of this transporter can alter Ca^{2+} homeostasis in tomatoes (Hirschi et al., 1996; Hirschi, 1999). Given the Ca^{2+} deficiency-like symptoms caused by *sCAX1* expression in tobacco using the cauliflower mosaic virus (CaMV) *35S* promoter (Hirschi, 1999), we opted to express *sCAX1* under the control of the *cdc2a* promoter (Doerner et al. 1996; Fig. 2A). In Arabidopsis, *cdc2a* transcript levels are correlated with the competence to divide; however, the expression of this Arabidopsis promoter has not been detailed in tomato. In our preliminary analysis, we found that *cdc2a*::*sCAX1* plants expressed less than half the amount of *sCAX1* RNA as compared to *35S*::*sCAX1* plants (data not shown). Initially we transformed *cdc2a*::*sCAX1* into tomato cv Red Cherry, a BER-tolerant variety. We use the abbreviation TCX1 (for tomato sCAX1) to identify plants expressing the *cdc2a*::*sCAX1* construct. A tomato vector control (TVC1)-expressing line is representative of the empty vector controls used in this study. We generated eight TCX1-expressing lines and eight TVC1-expressing lines. Initially, we examined the transgenic tomato lines by Southern analysis. TCX1 lines contain various

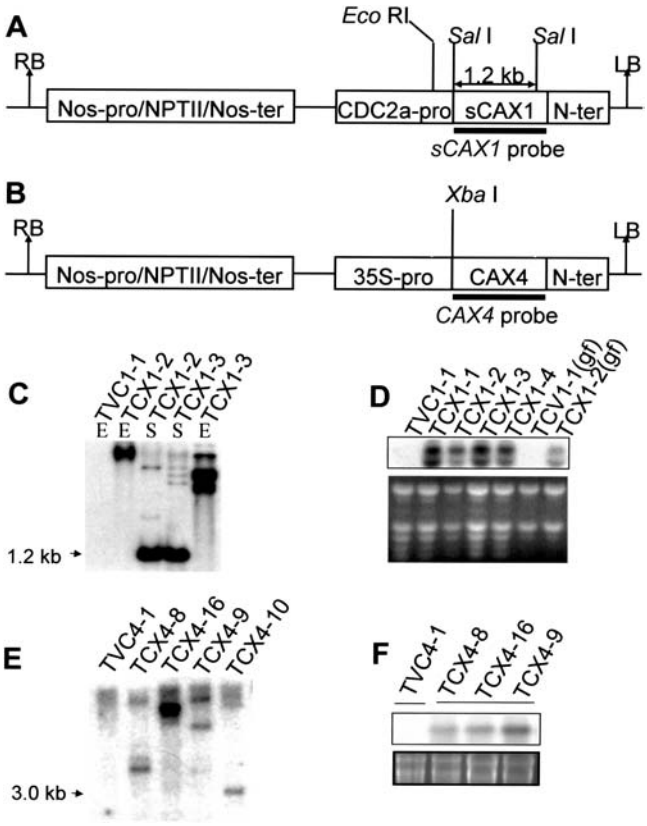

Figure 2. Molecular analyses of *sCAX1*- and *CAX4*-expressing tomatoes. A and B, T-DNA regions of pcdc2A::sCAX1 (A) and pCaMV35S::CAX4 (B). RB, Right border; LB, left border; Nos-pro, nopaline synthase promoter; NPTII, neomycin phosphotransferase; Nos-ter, nopaline synthase terminator; CDC2a-pro, cell division cycle promoter; 35S-pro, CaMV 35S promoter; CAX4, cation exchanger 4; N-ter, nopaline synthase terminator. C and E, Southern-blot analysis of transgenic tomato plants. Five to 10 μg of tomato genomic DNA were digested with *Eco*RI or *Sal*I (for pcdc2A::sCAX1) and with *Xba*I (for pCaMV35S::CAX4), and hybridized with the *sCAX1* cDNA probe (C) and *CAX4* cDNA probe (E), respectively. D and F, Northern-blot analysis of transgenic tomato plants. Ten micrograms of total RNA from expanded leaves and green fruit (gf) were hybridized with the *sCAX1* cDNA probe (D) and *CAX4* cDNA probe (F), respectively. Ethidium bromide-stained rRNA (bottom) is shown as a loading control.

copy numbers of the *sCAX1* expression construct (Fig. 2C). The line termed TCX1-2 contained a single insertion. The RNA gel blot documented that *sCAX1* transcripts accumulated in all TCX1 lines. We consistently saw two transcripts using the *cdc2a* promoter and only one with the *35S*::*sCAX1* tomatoes (Fig. 2D; data not shown). We attribute the two transcripts to multiple transcriptional initiation sites on the vector. The lower band of the two corresponded to the band seen in *35S*::*sCAX1* plants (data not shown). The inability to detect a tomato CAX1 homolog in the TVC lines by either Southern or northern blots may be due to the stringency of hybridization used in this study.

The *35S*::*CAX4* construct, in which *CAX4* was driven by the CaMV *35S* promoter (Fig. 2B), was transformed into tomato (cv Rubion). Ten independent *CAX4*-expressing lines were generated. We randomly selected four transgenic lines, and the stable integration and transmission of the *35S*::*CAX4* chimera in the genome of T_1 TCX4 tomatoes was confirmed by Southern-blot analysis (Fig. 2E). The lines we have termed TCX4-8, TCX4-9, and TCX4-10 appeared to contain more than one integration event, while line TCX4-16 had a single-copy insertion. RNA gel blot documented that *CAX4* transcripts accumulated in all of the transgenic lines (Fig. 2F; data not shown).

Three T_1 transgenic lines (TCX4-8, TCX4-9, and TCX4-16) showing a low copy number of the *CAX4* gene were selected and subjected to further analysis of Ca^{2+} accumulation and shelf life in *CAX4*-expressing fruits.

sCAX1 Expression Altered Tomato Growth

The expression of *sCAX1* caused all the tomato plants to be more compact and sturdier throughout their lives (Fig. 3, A and B). *sCAX1* expression caused

Figure 3. Phenotypes of *sCAX1*- and *CAX4*-expressing tomato plants. A, Growth of TVC1-1 vector control (left) and the *sCAX1*-expressing TCX1-2 plant (right) after 3 weeks in soil. B, Growth of vector control (left) and *sCAX1*-expressing TCX1-2 plant (right) after 5 months in soil. C, Growth of vector control (left) and *CAX4*-expressing TCX4-16 plant (right) after 4 months in soil. D, *sCAX1*-expressing TCX1-2 tomato grown in soil for 2 months without application of exogenous Ca^{2+} (left) and with the addition of Ca^{2+} (right). E, Root structure of vector control (left) and *sCAX1*-expressing TCX1-2 plant (right). F, Leaf phenotype of vector control (left) and *sCAX1*-expressing TCX1-4 plant (right).

necrotic lesions to form on primary transformants (Fig. 3D, left). This phenotype was apparent on all the *sCAX1*-expressing transformants, while none of the vector control lines displayed this phenotype. With the addition of Ca^{2+} to the growth media, the primary *sCAX1*-expressing transformants no longer had extensive apical burning (Fig. 3D, right). The *sCAX1*-expressing plants grown in soil also manifested mild Ca^{2+} deficiency-like symptoms; again, this phenotype could be significantly suppressed by adding Ca^{2+} to the watering solution. Examination of the root structure showed a 40% increase in the root mass in all of the *sCAX1*-expressing plants (root weight at day 40 was 34 ± 3 g for TCX1 lines, right, and 25 ± 3 g for TVC1 lines, left; Fig. 3E). Examination of plant height for the *sCAX1*-expressing plants (plant height from soil surface to the upper leaf; 105 ± 15 cm for TCX1 lines) found that they were approximately 50-cm shorter than vector control plants (165 ± 23 cm for TVC1 lines) after 5 months of growth in soil (Fig. 3B). The mature *sCAX1*-expressing plants appeared to have thicker leaves (0.4 ± 0.05 mm for TCX1 lines, right, and 0.2 ± 0.05 mm for TVC1 lines, left; Fig. 3F). The morphology of the sepals of *sCAX1*-expressing lines (right) was also altered compared to vector control lines (left; Fig. 4A). The fruit set was delayed by approximately 4 to 5 weeks in six of the *sCAX1*-expressing lines; the other two lines did not produce fruit. The overall shape of the transgenic fruit was indistinguishable from that of vector controls (Fig. 4, B, C, and E). However, the seed size was significantly reduced in the *sCAX1*-expressing lines compared to vector control lines (Fig. 4, B–D). One out of six *sCAX1*-expressing primary transformants was capable of making viable seed, in contrast to most of the vector control lines. Another striking phenotype of the *sCAX1*-expressing lines was a significantly increased occurrence of BER. The incidence of BER was higher in all of the *sCAX1*-expressing lines (75% ± 14% for TCX1 lines and 8% ± 5% for TVC1 lines; Fig. 4, I and J). *cdc2a*::*sCAX1* was also transformed into tomato cv FM9, another BER-tolerant variety, to determine whether the *sCAX1*-induced phenotypes were not a consequence of the Red Cherry variety used in the study. Severe BER symptoms were also observed for the FM9 variety expressing *sCAX1* in addition to the other changes in plant morphology that were found in *sCAX1*-expressing Red Cherry tomato lines (data not shown).

All subsequent experiments with *sCAX1*-expressing Red Cherry tomato lines were done on primary transformants that had similar phenotypes. The *cdc2a*::*sCAX1*-expressing primary transformants (TCX1-2 and TCX1-4), which have a single copy of *CAX1*, were used for all other analysis.

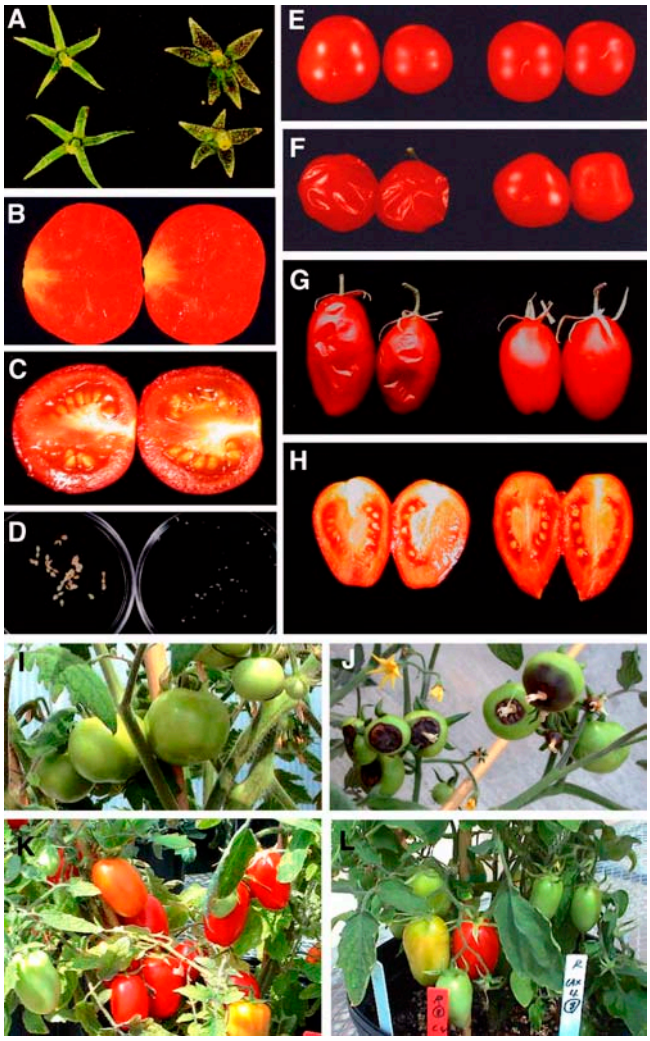

Figure 4. Phenotypes of *sCAX1*- and *CAX4*-expressing tomato fruits. A, Sepals of vector control (left) and *sCAX1*-expressing plants (right). B and C, Tomato longitudinal section of *sCAX1*-expressing tomato fruit (B) and vector control (C). D, Seeds of vector control (left) and *sCAX1*-expressing plants (right). E, Fruit from vector control (left) and *sCAX1*-expressing TCX1-2 and TCX1-4 plants (right) 20 d after breaker stage. F, Fruits from vector control (left) and *sCAX1*-expressing TCX1-2 and TCX1-4 plants (right) 40 d after breaker stage. G, Fruits from vector control (left) and *CAX4*-expressing TCX4-9 and TCX4-16 plants (right) 30 d after breaker stage. H, Tomato longitudinal section of vector control (left) and *CAX4*-expressing tomato fruit (right). I and J, BER of tomato fruits from vector control (I) and *sCAX1*-expressing tomato plant (J). The incidences of BER were mainly seen in the *sCAX1*-expressing plants. K and L, BER of tomato fruits from vector control (K) and *CAX4*-expressing tomato plant (L). No incidences of BER were mainly seen in the *CAX4*-expressing plants.

Phenotypes of *CAX4*-Expressing Tomatoes

While the *sCAX1*-expressing lines were sensitive to Ca^{2+} deficiency and showed Ca^{2+} deficiency-like symptoms that were suppressed by addition of Ca^{2+}, the *CAX4*-expressing lines were not sensitive to Ca^{2+} deficiency and did not require any additional Ca^{2+} supplementation for normal growth. In addition, both *CAX4*-expressing lines and wild-type control grew similarly on one-half strength Murashige and Skoog medium, Ca^{2+}-depleted one-half strength Murashige and Skoog medium, and the one-half strength Murashige and Skoog medium supplemented with various

ion metals, such as NaCl and $MgCl_2$ (data not shown). In TCX4 plants, *CAX4* expression did not perturb the morphology, growth (Fig. 3C), or fruit set (Fig. 4, K and L). Moreover, the fruit set was not delayed, all CAX4 transformants were capable of making viable seed (Fig. 4H), and the total fruit yield of CAX4 plants was indistinguishable from the wild-type control (data not shown). As the expression of *sCAX1* caused a significant increase in BER occurrence in supposed BER-tolerant tomato varieties (Red Cherry and FM9), we chose to express *CAX4* in a BER-susceptible tomato variety (cv Rubion) in order to examine the effect of *CAX4* expression on BER induction. The incidence of BER was equivalent in the *CAX4*-expressing lines (BER ratio; 15% ± 5% for TCX4 lines) and vector control lines (BER ratio; 18% ± 6% for TVC4 lines).

H^+/Ca^{2+} Antiport in *sCAX1*-Expressing Tomatoes

Expression of *sCAX1* in yeast restores H^+/Ca^{2+} antiport activity to yeast strains deficient in this transporter (Hirschi et al., 1996). In plants, expression of *sCAX1* in tobacco also causes increased H^+/Ca^{2+} antiport (Hirschi, 1999). H^+/Ca^{2+} antiport activity was measured in tonoplast-enriched vesicles from sCAX1 transgenic and vector control tomato roots to confirm that expression of *sCAX1* resulted in increased ΔpH-dependent Ca^{2+} transport. The *sCAX1* transcript was found to be constitutively expressed throughout the tomato plant in all lines (data not shown); therefore, root tissue was used to determine antiport activity. A proton gradient (acid inside vesicles) was formed by activation of the Mg^{2+}ATP-dependent H^+-ATPase. H^+/Ca^{2+} antiport activity measured in the presence of the P-type ATPase inhibitor, vanadate, was significantly higher in vesicles from the TCX1-2 line compared to the TVC1 (Fig. 5B). A TVC line accumulated Ca^{2+} to approximate steady-state concentrations of 0.22 ± 0.01 nmol Ca^{2+} mg protein^{-1} (Ca^{2+} uptake in the presence of vanadate without carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone [FCCP] was 0.93 ± 0.01 nmol Ca^{2+} mg protein^{-1} and in the presence of vanadate with FCCP was 0.71 ± 0.01 nmol Ca^{2+} mg protein^{-1}), while TCX1-2 accumulated Ca^{2+} to concentrations of 0.39 ± 0.09 nmol Ca^{2+} mg protein^{-1} (Ca^{2+} uptake in the presence of vanadate without FCCP was 1.2 ± 0.08 nmol Ca^{2+} mg protein^{-1} and in the presence of vanadate with FCCP was 0.81 ± 0.02 nmol Ca^{2+} mg protein^{-1}). Ca^{2+} transport activity measured in the absence of vanadate and in the presence of the protonophore FCCP was identical in vesicles from both plants (Fig. 5A).

Ca^{2+} Accumulation in *CAX*-Expressing Tomato Plants

Altered H^+/Ca^{2+} antiport activity in tonoplast-enriched membrane vesicles from *sCAX1*-expressing tomato plants indicated perturbed Ca^{2+} transport properties. To ascertain whether *sCAX1* expression

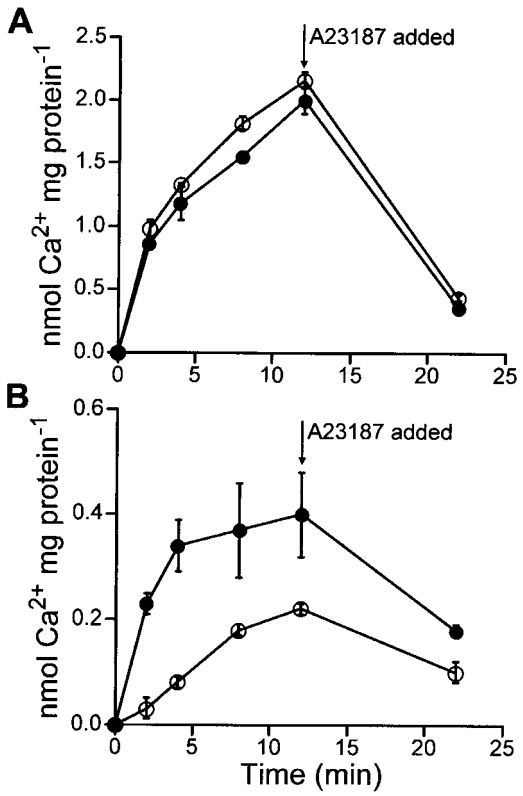

Figure 5. Ca^{2+} uptake activity into tonoplast-enriched vesicles from tomato root. A and B, Time courses of Mg^{2+}ATP-energized 10 μM $^{45}Ca^{2+}$ uptake were performed in the presence of 0.1 mM NaN_3, 10 mM KCl, 3 mM ATP, and 3 mM $MgSO_4$. Ca^{2+}-ATPase activity (A) was determined in the absence of orthovanadate and the presence of 5 μM FCCP. ΔpH-dependent H^+/Ca^{2+} antiport activity (B) was determined in the presence of 0.2 mM orthovanadate as the difference between Ca^{2+} uptake in the absence and presence of 5 μM FCCP. The Ca^{2+} ionophore A23187 (5 μM) was added at the time indicated. Black circles, sCAX1-expressing TCX1-2 plant; white circles, vector controls. Results are the average (± SE) of six replicate experiments using two independent membrane preparations.

altered ion levels, Ca^{2+} levels and other minerals were measured in the transgenic plants. The leaves of all *sCAX1*-expressing plants showed at least a 20% increase in Ca^{2+} levels (data not shown), while initial measurements of fruit Ca^{2+} levels showed that three (TCX1-2, -3, and -4) of the *sCAX1*-expressing lines had significant alteration in Ca^{2+} (Fig. 6A). Over time the TCX1-2 and TCX1-4 fruits contained more than twice the Ca^{2+} levels as vector control plants that were supplemented with Ca^{2+} (Fig. 6B). TCX1-2 and TCX1-4 fruits also contained increased levels of Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} (Fig. 6, C and D). Similar changes were seen in the other *sCAX1*-expressing line (TCX1-3), which contains multiple copies of *sCAX1* (data not shown).

All of the *CAX4*-expressing T_1 tomatoes (TCX4-8, -9, and -16) contained significantly more Ca^{2+} (40%–50% increase) than vector controls (Fig. 6E). No significant increase of other minerals (Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, and

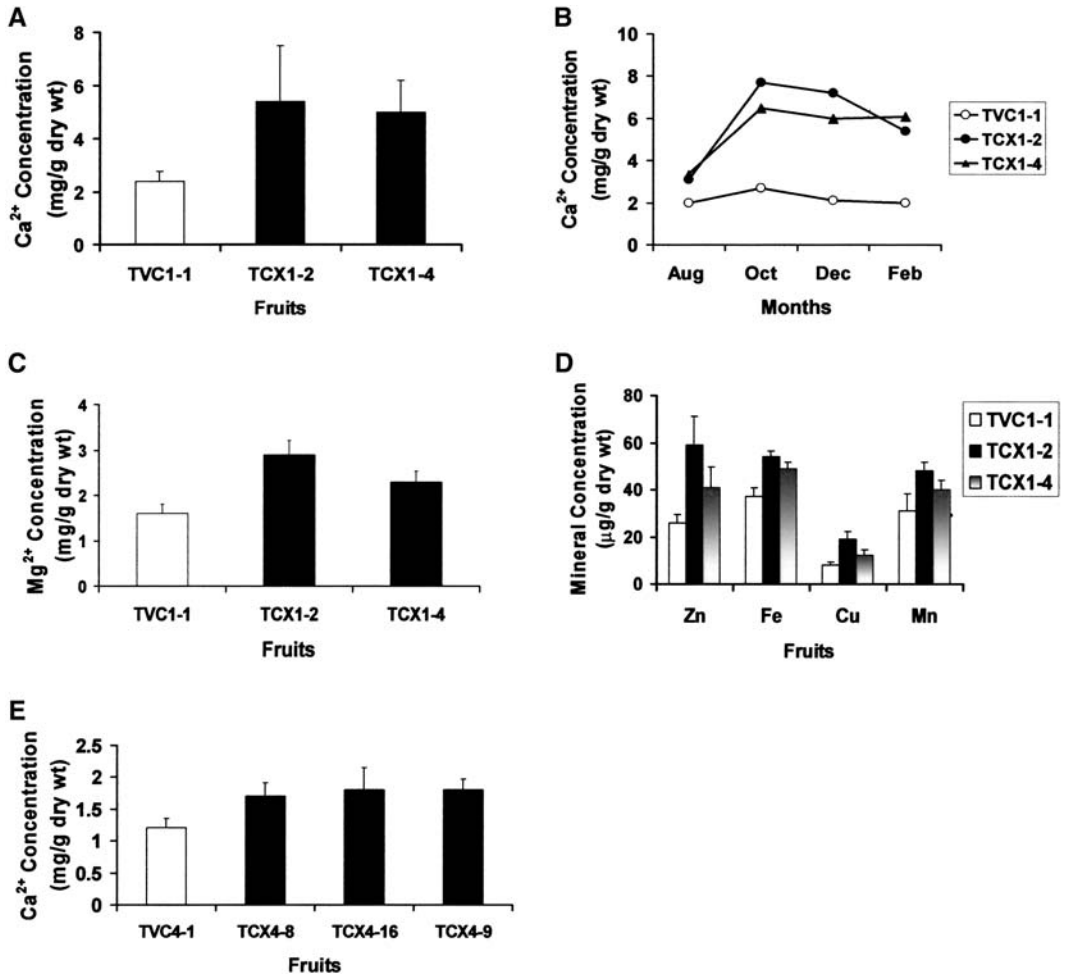

Figure 6. Concentrations of Ca^{2+} and other minerals in fruits of *sCAX1*- and *CAX4*-expressing plants. A to E, Fruit Ca^{2+} and mineral analysis was performed at 20 d after breaker stage. Total Ca^{2+} (A, B, and E) and mineral contents (C and D) of fruits (pooled at least five-fruit batches) were determined by inductively coupled plasma emission spectrophotometer. Data represent the values obtained from pools containing at least five fruit over a 6-month period (B), and the means (±SD) of four independent analyses (A and C–E). E, Concentrations of Ca^{2+} in fruits of *CAX4*-expressing plants.

Zn^{2+}) was observed with any of the lines analyzed (data not shown). In addition, no significant differences were observed for Na^+ and K^+ levels in both *sCAX1*- and *CAX4*-expressing lines compared to wild-type lines (data not shown).

Increased Fruit Firmness and Prolonged Shelf Life in *CAX*-Expressing Plants

In some agriculturally important crops, addition of exogenous Ca^{2+} increases fruit firmness and shelf life (Lester and Grusak, 1999; Lopez-Lefebre et al., 2001). We were interested in determining whether the increased Ca^{2+} in the transgenic fruit would cause increased firmness in the TCX1 lines. As shown in Figure 7, the decline in fruit firmness that is associated with fruit ripening was greatly delayed in *sCAX1*-expressing plants. During the first 30 d after the breaker stage, the TCX1 fruits were twice as firm as the controls. After 40 d, tomato fruits harvested from *sCAX1*-expressing plants maintained their structural integrity, while the vector control lines were significantly shrunken (Fig. 4F). Similar changes were seen in the *sCAX1*-expressing line (TCX1-3) containing multiple copies of the transgene (data not shown).

To determine whether the increased Ca^{2+} in the transgenic fruit increased firmness and prolonged shelf life in the TCX4 lines, we measured the mean separations at specific time points (i.e. 5, 10, 15, 20, 25, 30, 35, and 40 d). A significant change was observed in break date and treatment (Table I). At 30 d after the breaker stage, the TCX4 fruits harvested from *CAX4*-expressing plants maintained their structural integrity, while the vector control fruits were significantly shrunken (Fig. 4G). Overall, the decline in fruit firmness that is associated with fruit ripening was slightly delayed in *CAX4*-expressing plants (data not shown), and the shelf life of *CAX4*-expressing T_2 tomatoes extended the time until shrinkage about 5 d compared to the vector controls (Table I).

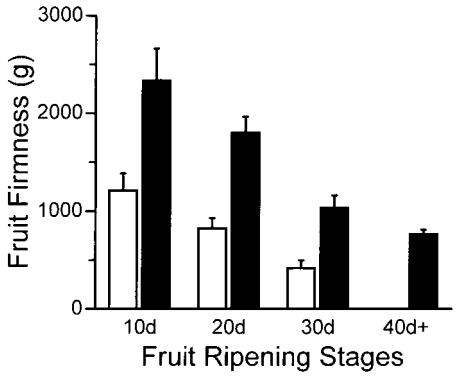

Figure 7. Firmness analysis in fruits of *sCAX1*-expressing plants. Fruits were harvested at 10 (10d), 20 (20d), 30 (30d), and 40 (40d) days after the first color change (the breaker stage). Four fruits were used for each measurement. Firmness was determined using a texture analyzer. Values shown are the means (±SD) of four fruits. White bars, control; black bars, fruits of *sCAX1*-expressing plants.

Sugar Concentration and Ethylene Production

Because the phenotypes were the most prevalent in the *sCAX1*-expressing plants, we measured sugar and ethylene levels in these transgenic fruit during ripening. The sugar concentration and ethylene production in the *sCAX1*-expressing lines were comparable to those of the vector control lines (Fig. 8, A and B). Again, similar results were obtained in two *sCAX1*-expressing lines having a single copy (TCX1-4) and multiple copies (TCX1-3) of the transgene (data not shown). Like the *sCAX1*-expressing fruit, the *CAX4*-expressing fruit has no alteration in sugar concentration compared to wild-type (data not shown).

DISCUSSION

We selected tomato as the vegetable in which to express H^+/Ca^{2+} transporters as a means to increase Ca^{2+} content. Three tomato cultivars, Red Cherry and FM9, fresh market tomatoes reported to have some tolerance to BER (K. Crosby, Texas Agricultural Experiment Station, personal communication), and Rubion, a processing tomato completely susceptible to BER (R. Johns, Seminis, personal communication), were selected in this study. Ca^{2+} deficiency is the most common nutritional problem affecting tomatoes, which comprise the second-largest vegetable crop in the United States, after potatoes. A lack of Ca^{2+}, water, or both can cause BER in tomatoes (Ho and White, 2005). In addition, tomatoes are highly susceptible to postharvest decay, and must be handled with special care to avoid wounds, bruises, and other injuries that would serve as ports of entry for various pathogens. To ensure firmness on arrival after shipping, tomatoes are picked at the mature green stage, which precludes extended vine ripening. Here we have demonstrated the ability to increase Ca^{2+} levels in tomatoes, which may be a means to deliver more Ca^{2+} to consumers, adjust growth constraints, and vary the harvesting practices of an agriculturally important commodity.

Previous studies have demonstrated that expression of Ca^{2+}-signaling components, such as a Ca^{2+} transporter or a Ca^{2+}-binding protein, can be used to increase Ca^{2+} levels in various plants (Wyatt et al., 2002; Hirschi, 2004). In this study, we have expressed *sCAX1* driven by the cell cycle promoter *cdc2a* (Doerner et al., 1996; Fig. 2A). To our knowledge, the Arabidopsis *cdc2a* promoter has not been previously used in tomato and here we show the first preliminary examination of its use in this plant. The *cdc2a*-driven *sCAX1* expression was found to be constitutively expressed in all tomato tissues analyzed (data not shown), suggesting that sCAX1 H^+/Ca^{2+} antiport activity would be equally enhanced in all tissues compared to control plants. We therefore chose to measure antiport activity from root tissue rather than tomato fruit as it is technically much more difficult to isolate intact membrane vesicles from fruit, particularly when ripe, rather than from other tissues. Roots from TCX1 plants showed a 44% increase in tonoplast-enriched H^+/Ca^{2+} transport (Fig. 5B) and no change in Ca^{2+}-ATPase activity (Fig. 5A). These results show we have not measurably perturbed another Ca^{2+} transport mechanism on the root vacuolar membrane.

The *cdc2a*::*sCAX1*-expressing tomatoes without additional Ca^{2+} supplementation in the soil demonstrate

Table I. *Shelf life analysis of CAX4-expressing tomato fruits*

The letters (a, b, c, and d) denote statistically significant differences between TCX4 fruits and control fruits. One balanced design (12 plots) and one unbalanced design (eight plots) containing each of 20 fruits a plot were used for shelf life analysis by a randomized-complete block design with three replications. Data were analyzed by ANCOVA, and mean separations was based on the Tukey-Kramer procedure at $\alpha = 0.05$.

CAX4-Expressing Lines (Treatment)	n[a]	Shrinkage Rating Scales[b] after Breaker Stage						
		Break Date						
		0	5	10	15	20	25	30
TVC4-1 (Control)	120	0.0a	0.2a	0.8a	1.3a	1.9a	2.5a	3.0a
TCX4-8	60	0.0a	0.1b	0.6b	1.2b	1.7b	2.3b	2.8b
TCX4-9	100	0.0a	0.0b	0.4c	0.9c	1.4d	1.9d	2.4d
TCX4-16	120	0.0a	0.0b	0.6b	1.1b	1.6c	2.1c	2.6c

[a]Total number of fruits tested. Fruits were harvested at mature green stage and ripened in air at 22°C to 24°C. [b]Shrinkage rating scales were as follows: rate 1, signs of shrinkage; rate 2, ≤25%; rate 3, 25% to 50%; and rate 4, >50%.

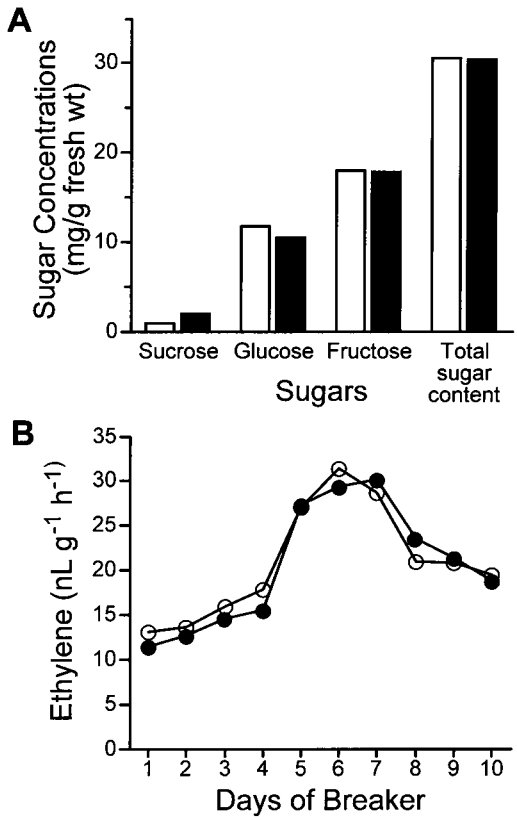

Figure 8. Sugar and ethylene production in fruits of *sCAX1*-expressing plants. A, Concentration of total sugars in fruits of *sCAX1*-expressing plants. Control (30.60 mg g^{-1} fresh weight and *sCAX1*-expressing fruits (30.54 mg g^{-1} fresh weight) were harvested at 20 d after breaker stage, and the concentration of soluble sugars in fruits (pooled four-fruit batches) were analyzed by HPLC. Values (Suc, Glc, and Fru) shown are the means of three injections on the HPLC. White bars, control; black bars, fruits of *sCAX1*-expressing plants. B, Ethylene production was assayed during a 10-d ripening period after initiation of the first color change. Data shown are the means of values obtained from three fruits. White circles, control; black circles, fruits of *sCAX1*-expressing plants.

symptoms of Ca^{2+} deficiencies, particularly apical burning (Fig. 3D, left). The alterations in plant size, leaf morphology, fruit set, and ripening (Figs. 3 and 4) further emphasize the importance of regulated Ca^{2+} transport in plant growth and development. Several of these phenotypes, particularly the apical burning, are similar to those of *sCAX1*-expressing tobacco plants (Hirschi, 1999). Thus, despite the increased Ca^{2+} in both tobacco and tomato plants, they are suffering from Ca^{2+} deficiency symptoms (see below). Ca^{2+} supplementation in the soil (Fig. 3D, right) was required to obtain maximal growth of *sCAX1*-expressing tomato plants (Fig. 3, D and F). The roots of the *cdc2a*::*sCAX1*-expressing tomatoes appeared more vigorous than those of the control plants. These findings implicate the levels of Ca^{2+} in strongly influencing root growth (Picchioni et al., 2001). This is interesting when one considers that *35S*::*sCAX1* expression in tobacco decreases root mass (Hirschi, 1999). Our findings suggest that the use of the *cdc2a* promoter mitigated some of the most severe symptoms associated with high-level expression of *sCAX1*. Some cation/H^+ antiporters are known to have a role in regulating cytoplasmic or vacuolar pH (Yamaguchi et al., 2001). We have not analyzed the effect of CAX expression on pH levels in the tomato, and it will be interesting to see whether any morphological changes to the plants are due to altered pH homeostasis.

Unexpectedly, one of the most deleterious changes in fruit development caused by *sCAX1* expression in the tomato cultivars, Red Cherry and FM9 (both cultivars have been reported to have tolerance to BER), was the dramatically increased incidence of BER (Fig. 4J). Evidence for Ca^{2+} deficiency as the primary cause of BER has been derived from observations that the blossom end has the lowest content of Ca^{2+} within tomato fruits (Adams and Ho, 1993; Nonami et al., 1995). BER is generally associated with the disintegration and increased ion permeability of cells, resulting in loss of turgor and cell fluids invading the intercellular air space, thus causing the watery appearance in the early stages (Shear, 1975; Simon, 1978). However, despite the increased Ca^{2+} in tomato fruits, the increased incidence of BER in *sCAX1*-expressing tomatoes is perplexing. We expect that the increased Ca^{2+} in the tomato fruit is due to a significant increase in vacuolar Ca^{2+} level within the rapidly expanding fruit due to the enhanced and unregulated vacuolar Ca^{2+}/H^+ antiport activity. Therefore, one possible explanation for the increased BER is that *sCAX1*-expressing lines have altered Ca^{2+} homeostasis between cytosolic, apoplastic, and vacuolar Ca^{2+} pools, with decreases in cytosolic and apoplastic Ca^{2+} levels. It has been suggested that this may disrupt Ca^{2+}-signaling processes, membrane integrity, and normal cell wall development, leading to cell death and thus the occurrence of BER (Ho and White, 2005). Future work will therefore need to determine whether there is indeed reduced cytosolic and/or apoplastic Ca^{2+} levels in the *sCAX1*-expressing tomato fruit. An alternative explanation for increased occurrence of BER might be that expression of *sCAX1* in vegetative tissues results in a localized Ca^{2+} deficiency in early fruit development.

While the *sCAX1*-expressing Red Cherry and FM9 (BER-tolerant cultivars) have increased incidence of BER, no significant difference was observed with the *CAX4*-expressing Rubion (a cultivar susceptible to BER) when compared to vector controls (Fig. 4, K and L). This may be due in part to the more modest increase in tomato fruit Ca^{2+} levels seen with TCX4 lines compared to the TCX1 lines. Moreover, *CAX4* expression did not perturb the morphology, growth (Fig. 3C), or fruit set (Fig. 4L). Future work will be focused on the mechanisms of *CAX* expression and BER development.

The deleterious changes in plant growth caused by *cdc2a*::*sCAX1* expression in tomato plants (e.g. Fig. 3, B and E) suggest that further modulation of the expression of H^+/Ca^{2+} transporters is needed. Rather than alter the expression of the transporter, another

approach is to turn down the CAX-mediated Ca^{2+} transport throughout the plant by posttranslational down-regulation. Here we have used full-length CAX4 containing the entire putative N-terminal autoinhibitory domain. CAX4 is 54% identical to CAX1 at the amino acid level, and previous work suggests that repositioning of the N terminus in this transporter confers Ca^{2+} transport in yeast assays (Fig. 1A; Cheng et al., 2002). When HA-CAX4 is expressed in yeast, competition experiments suggest that this variant is a more specific Ca^{2+} transporter than sCAX1 or the variant of CAX4 that contains the Ca^{2+} domain of CAX1 (sCAX4-9). However, it is possible that the presence of the HA tag could disrupt the selectivity or the regulatory properties of CAX4. Transport experiments with sCAX4 should determine whether the HA tag does actually affect CAX4 activity. Unfortunately we were unable to obtain transport measurements for sCAX4 in the yeast system so alternative approaches will be required to address this question in the future. Our working hypothesis is that in planta CAX4 is an H^+/Ca^{2+} transporter. Interestingly, we attempted several times to generate transgenic lines expressing either sCAX4-9 or HA-CAX4 but were unable to produce viable plants (data not shown).

While the *cdc2a*::*sCAX1* tomato plants have increased Ca^{2+} when compared to vector controls, they also display increased levels of several other ions, particularly Mg^{2+}, Zn^{2+}, Fe^{2+}, and Mn^{2+} (Fig. 6). Metal competition experiments infer that sCAX1 can transport Cd^{2+} as well as Ca^{2+} but not Mn^{2+} (Fig. 1B; Shigaki et al., 2001). Competition experiments also suggest that sCAX1 cannot transport Fe^{2+} and Cu^{2+} (data not shown), while direct transport measurements have shown that wild-type sCAX1 cannot efficiently transport Ni^{2+}, Co^{2+}, and Mn^{2+} but has some ability to transport Cd^{2+} and Zn^{2+} in addition to Ca^{2+} (Shigaki et al., 2005). Similar direct transport experiments will be needed to further determine the transport properties of CAX4 and CAX4 variants. Meanwhile, in agreement with the yeast uptake assays using HA-CAX4, the *CAX4*-expressing plants have increased levels only of Ca^{2+}. The lack of deleterious morphological phenotypes in the *CAX4*-expressing plants may correlate with the absence in accumulation of various transition metals. Even at micromolar concentrations some transition metals can be particularly toxic (Marschner, 1995); therefore, some of the phenotypes in the *sCAX1*-expressing plants could also be due in part to toxicity induced by Fe^{2+}, Zn^{2+}, or Mn^{2+} accumulation. It is also worth noting that increased H^+/Ca^{2+} transport activity may activate other proton-mediated transporters (Cheng et al., 2005) to cause alterations in plant growth.

While all the *sCAX1*-expressing lines showed increased Ca^{2+} levels in the leaves (data not shown), only a portion of the lines showed changes in Ca^{2+} levels in the fruit. This may be because we did not assay fruit from older plants for all the lines. Our data suggest that fruit derived from older *sCAX1*-expressing plants contain more Ca^{2+} (Fig. 6B). Alternatively, the sCAX1 transporter may have to be highly expressed in particular cells in order to facilitate increased Ca^{2+}.

Currently, most Americans obtain their dietary Ca^{2+} from milk-related products; fruits like tomatoes do not contribute significantly to Ca^{2+} intake (Fleming and Heimback, 1994). In other areas of the world, communities obtain a majority of their total dietary Ca^{2+} from the consumption of fruits and vegetables (Weaver et al., 1999). Increasing the endogenous levels of Ca^{2+} in commonly consumed fruits should help yield improved dietary Ca^{2+} intakes within many population groups. However, bioavailability studies are needed to determine to what extent these mineral changes in fruit translate into improved Ca^{2+} bioavailability and nutritional quality.

Applications of $CaCl_2$ are used as a means to increase the firmness of various fruits prior to shipment. Since little translocation of Ca^{2+} occurs from leaves to growing fruits, direct Ca^{2+} application on the surface is recommended. However, late-season application of Ca^{2+} to apples (*Malus domestica*), pears (*Pyrus communis*), and other commodities is often avoided due to the costs and the potential of damaging the fruit or accelerating postharvest fungal infections. We have demonstrated here that expression of CAX transporters in tomatoes can be used to increase the firmness and shelf life of tomato fruit (Figs. 4, F and G, and 7). Interestingly, *sCAX1* expression does not appear to alter the sugar concentration of the tomatoes (Fig. 8). These findings imply that CAX-expressing tomatoes can be left on the vine to ripen longer, enabling them the potential to offer improved flavor while retaining the firmness necessary to withstand the rigors of shipping. Exogenous applications of Ca^{2+} to fruit have been shown to be associated with decreased fruit respiration rate and ethylene production (Faust and Shear, 1972; Lieberman and Wang, 1982). However, an increase in fruit Ca^{2+} levels by enhanced activity of vacuolar Ca^{2+}/H^+ antiport does not appear to decrease ethylene production (Fig. 8B). The mechanisms by which Ca^{2+} effects ethylene biosynthesis are not fully understood, but it is conceivable that cytosolic Ca^{2+} signals may have a role in regulating ethylene production (Njoroge et al., 1998). While exogenous application of Ca^{2+} to tomato fruit will likely cause an initial increase in cytosolic Ca^{2+} and a decrease in ethylene, increase in fruit Ca^{2+} by *sCAX1* overexpression will increase vacuolar Ca^{2+} levels but may prevent any substantial increase in cytosolic Ca^{2+}, thus preventing a decrease in ethylene production.

Tomato fruit is a climacteric fruit in which ripening is initiated by increased production of ethylene (Adams-Phillips et al., 2004; Giovannoni, 2004). In this study we have observed that the fruit from *sCAX1*-expressing plants ripen more slowly (Figs. 4F and 7) despite ethylene production being unperturbed (Fig. 8B). Previous studies have also demonstrated alteration in tomato fruit ripening without an alteration in ethylene biosynthesis. The tomato mutants *Green-ripe*, *Never-ripe* (*Nr*), and *Nr-2* do not fully ripen, yet ethylene synthesis

is unchanged compared to wild-type fruit (Lanahan et al., 1994; Barry et al., 2005). It was suggested that these altered-ripening phenotypes are due to ethylene insensitivity. In fact, in the *Nr* mutant, this insensitivity is due to a mutation in an ethylene receptor (Wilkinson et al., 1995). Studies suggest that Ca^{2+} is involved in signaling pathways regulating fruit ripening. For example, it has been shown that Ca^{2+} is required for ethylene-dependent processes (Raz and Fluhr, 1992) and a tomato Ca^{2+}-dependent protein kinase-related kinase, LeCRK1, which is regulated during ripening has been identified (Leclercq et al., 2005). The results from our work suggest that altering the Ca^{2+} homeostasis of the fruit by *sCAX1* expression can affect the regulation of fruit ripening. Future work will need to be directed at the actual signaling and developmental events that are altered in the *CAX*-expressing plants, such as whether the plants are ethylene-insensitive like the *Nr* mutants.

In addition to the possible effect of increased Ca^{2+} on fruit ripening pathways, the excess Ca^{2+} may also enhance fruit firmness due to improved cell wall integrity. Cell wall-associated Ca^{2+} maintains cell wall integrity by generating cross-links with nonesterified pectins in the primary cell wall and middle lamella (Jarvis, 1984; Poovaiah et al., 1988). Homogalacturonan, one of the main polymers of pectin, can form cross-links with Ca^{2+} by the binding of Ca^{2+} with carboxylate ions, to form parallel and antiparallel chains, causing the wall to be more rigid. The presence of Ca^{2+}-pectin bridges prevents the access of cell wall hydrolytic enzymes, thus inhibiting cell wall expansion (Jauneau et al., 1994; Konno et al., 1999). Further work is required to demonstrate that cell wall-associated Ca^{2+} levels are indeed enhanced in CAX-expressing plants compared to wild type.

Increased Ca^{2+} levels have been shown to alter the severity of several plant pathogens (Marschner, 1995). In the *sCAX1*-expressing tomatoes, we see no alterations to infection by *Pseudomonas syringae* pv tomato and *Xanthomonas campestris* pv vesicatoria or Botrytis (data not shown). Further tests must be performed in various environmental conditions to firmly establish that we have not altered the susceptibility to pathogens. However, it is easy to envision that increasing the firmness of the tomato fruits could delay the penetration of a particular pathogen.

In this report, we have demonstrated the ability to increase Ca^{2+} levels in tomatoes through heightened activity of a Ca^{2+} transporter. We have demonstrated here that expression of the H^+/Ca^{2+} transporters can increase fruit Ca^{2+} levels as well as firmness.

MATERIALS AND METHODS

Yeast Growth, Vacuolar Membrane Isolation, and Transport Measurements

The yeast (*Saccharomyces cerevisiae*) strain K667 (*cnb1*::*LEU2 pmc1*::*TRP1 vcx1*Δ) (Cunningham and Fink, 1996) was used to express various CAX cDNA constructs in the yeast shuttle vector pHGpd (Nathan et al., 1999). Yeast transformation using the lithium acetate/polyethylene glycol transformation method, and isolation of yeast vacuolar-enriched membrane vesicles, were performed as described previously (Pittman et al., 2004a). Measurements of $^{45}CaCl_2$ uptake into yeast membrane vesicles and metal competition experiments were performed as described previously (Shigaki et al., 2003).

Plant Material, Transformation, and Growth Conditions

Seeds of tomato (*Lycopersicon esculentum*) Mill. cultivars Red Cherry, FM9, and Rubion were surface sterilized. Seeds were germinated on a Murashige and Skoog (1962) inorganic salt medium with 30 g L^{-1} Suc, pH 5.7, and solidified using 8 g L^{-1} TC agar (Sigma). Tomato transformation was performed via Agrobacterium-mediated transformation method using cotyledon and hypocotyl explants as described (Park et al., 2003). Cultures were maintained at 25°C under a 16-h photoperiod. After 6 to 8 weeks (subcultured once at 3–4 weeks), regenerated shoots were transferred to rooting medium for 6 more weeks (for *sCAX1*-expressing transgenic lines, 3 mL of 2 mM $CaCl_2$ were added to rooting medium to suppress the growth defects of *sCAX1*-expressing tomato once at 4 weeks), then established in soil. All plants were watered as needed. Once a week they were watered with Miracle-Gro for tomato (Scotts Miracle-Gro Products). The temperature of the greenhouse was maintained within a range of 25°C to 30°C. For maximal growth, *sCAX1*-expressing transgenic lines in the greenhouse were watered to saturation with 2 mM $CaCl_2$ twice a week for the first 8 weeks.

Bacterial Strain and Plasmids

Agrobacterium tumefaciens LBA 4404 octopine (Hoekema et al., 1983) was used for the transformations. The plasmid pcdc2A::sCAX1 or pCaMV35S::CAX4 was introduced into *A. tumefaciens* using the freeze-thaw method (Holsters et al., 1978). The *sCAX1* open reading frame was cloned into the *nos/nptII/nos-ter/cdc2a /nos-ter* expression vector, which was obtained from John Celenza (Doerner et al., 1996).

DNA Isolation and Southern-Blot Analysis

Tomato genomic DNA was extracted from leaf tissue as previously described (Paterson et al., 1983). DNA (5–10 μg) was digested with *Sal*I or *Eco*RI and separated by electrophoresis and blotted onto a nylon membrane (Zeta-probe GT membrane, Bio-Rad Laboratories) according to the manufacturer's instructions. The probe for the *sCAX1* gene was isolated from a *Not*I (1.4 kb) restriction fragment of the p039 plasmid (Hirschi et al., 1996), and the probe for the *CAX4* gene was isolated from a *Sac*I-*Xba*I (1.3 kb) restriction fragment of the pBlue-CAX4 plasmid (Cheng et al., 2002). The membranes were prehybridized overnight at 65°C in 7% SDS and 0.25 M Na_2HPO_4, and then hybridized overnight at 65°C in the same solution containing the probe labeled with ^{32}P-dCTP using NEBlot kit (NEB BioLabs). Membranes were washed twice for 30 min each with 20 mM Na_2HPO_4 and 5% SDS at 65°C and then washed twice again for 30 min each with 20 mM Na_2HPO_4 and 1% SDS at 65°C. Membranes were exposed to x-ray film at −80°C.

RNA Isolation and Northern-Blot Analysis

Total RNA was extracted from green fruit tissues and leaves using RNeasy plant kits (Qiagen) according to the manufacturer's instructions. Total RNA (7 μg) was separated on a 1.2% agarose gel containing 1.5% formaldehyde, blotted onto a Zeta-Probe GT membrane according to the manufacturer's instructions. Hybridization and washing were as previously described in Southern-blot analyses.

Plant Measurements

The heights of plants (from soil surface to the upper leaf) were measured after 5 months of growth in soil. The means (±SD) of three independent *sCAX1*-expressing lines were compared to the means (±SD) of three control lines. At this stage, five leaves of similar age from each of these three transgenic lines and three control lines were sampled, and leaf thickness was measured under a microscope (model 475050, Zeiss). Pictures of the leaf shape of these plant lines were taken to record the phenotypes.

Root Measurements

Root mass was measured by taking three TCX1-expressing lines and three TVC1-expressing lines and after 2 months of growth in soil gently removing the soil from the roots using water. The intact plants were then transferred to hydroponic growth conditions and allowed to grow for 14 additional days as previously described (Hirschi, 1999). The roots were then harvested and dried as previously described (Hirschi, 1999).

Endomembrane Isolation and Ca^{2+} Transport Assay

Roots of 4-week-old soil-grown TVC1 and TCX1-2 plants were homogenized at 4°C and microsomal pellets obtained (Hirschi, 1999). Endomembrane-enriched vesicles were then prepared as previously described (Pittman et al., 2002). The $^{45}Ca^{2+}$ transport assay was performed as previously described (Pittman and Hirschi, 2001) except 5 μM FCCP was used instead of gramicidin.

Ca^{2+} and Mineral Analysis

Fruit Ca^{2+} and mineral analysis was performed at 20 d after breaker stage, and the fruits (pooled at least five-fruit batches) were dried at 70°C for 4 d. A total of 0.25 g (dry weight) of fruits was digested for analysis (Feagley et al., 1994). Ca^{2+} and mineral contents per gram of dry weight were determined by inductively coupled plasma emission spectrophotometer (Spectro).

Firmness Analysis

Fruits were harvested at 10, 20, 30, and 40 d after the first color change (the breaker stage). Four fruits were used for each measurement. Firmness was determined using a TA-XTZi texture analyzer (Texture Technologies). A speed of 2 mm s^{-1} was used to compress fruit by 4 mm with a circular probe of 4.5 cm in diameter.

Shelf Life Analysis

After segregation analysis on T_2 seeds from self-pollinated T_1 plant lines (showing a segregation pattern of 3:1 on kanamycin medium), fruit from each of 10 homozygous T_2 lines was selected. Fruit was harvested at the mature green stage and ripened at 22°C to 24°C. One balanced design (12 plots) and one unbalanced design (8 plots) containing each of 20 fruits per plot were used for shelf life analysis by a randomized complete block design with three replications. After the breaker stage, the mean separations were performed at special break date values of 5, 10, 15, 20, 25, 30, 35, and 40 d. In this study, shrinkage rating scales were as follows: rate 1 (signs of shrinkage), rate 2 ($\leq$25%), rate 3 (25%–50%), and rate 4 (>50%). Data were analyzed by ANCOVA, and mean separations was based on the Tukey-Kramer procedure at $\alpha = 0.05$. Observation on fruit number and percentage of incidences of BER were recorded on all fruits from all plant lines selected in this study.

Ethylene Production Analysis

Ethylene production was assayed during a 10-d ripening period after the start of the first color change. Three fruits were used for each measurement. Individual fruits were placed into sealed containers at room temperature for 1 h, and then 1-mL gas samples were withdrawn. Gas samples were analyzed via gas chromatography (model 8500 gas chromatograph, Perkin-Elmer) using a 5% carbowax column (1.8 m $\times$ 2.1 mm) and a flame-ionization detection system.

Sugar Analysis

Standard sugar analysis was performed (Hamilton et al., 1997). Ten grams of fruits (pooled in four-fruit batches) at 20 d after the first color change were cut and blended with 80% ethyl alcohol and filtered.

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